Mol Gen Genet (1999) 262: 401–412

ORIGINAL PAPER

C. Mullins · L. M. Hartnell · D. A. Wassarman
J. S. Bonifacino

Defective expression of the μ3 subunit of the AP-3 adaptor complex in the Drosophila pigmentation mutant *carmine*

Received: 7 June 1999 / Accepted: 4 July 1999

Abstract The adaptor protein (AP) complexes AP-1, AP-2, and AP-3 mediate coated vesicle formation and sorting of integral membrane proteins in the endocytic and late exocytic pathways in mammalian cells. A search of the *Drosophila melanogaster* expressed sequence tag (EST) database identified orthologs of family members mammalian medium (μ) chain families μ1, μ2, and μ3, of the corresponding AP complexes, and δ-COP, the analogous component of the coatomer (COPI) complex. The Drosophila orthologs exhibit a high degree of sequence identity to mammalian medium chain and δ-COP proteins. Northern analysis demonstrated that medium chain and δ-COP mRNAs are expressed uniformly throughout fly development. Medium chain and δ-COP genes were cytologically mapped and the μ3 gene was found to localize to a region containing the pigmentation locus *carmine* (*cm*). Analysis of genomic DNA of the cm^1 mutant allele indicated the presence of a large insertion in the coding region of the μ3 gene and Northern analysis revealed no detectable μ3 mRNA. Light microscopy of the cm^1 mutant showed a reduction in primary, secondary, and tertiary pigment granules in the adult eye. These findings provide evidence of a role for μ3 in the sorting processes required for pigment granule biogenesis in Drosophila.

Key words Clathrin · Sorting · Tyrosine-based signal · Pigment · Hermansky-Pudlak syndrome

Communicated by J. A. Campos-Ortega

C. Mullins · L. M. Hartnell · D. A. Wassarman
J. S. Bonifacino (✉)
Cell Biology and Metabolism Branch,
National Institute of Child Health and Human Development,
Building 18T/Room 101, National Institutes of Health,
Bethesda, MD 20892, USA
e-mail: juan@helix.nih.gov
Tel.: +1-301-4966368, Fax: +1-301-4020078

Introduction

Protein trafficking between organelles of the secretory and endocytic pathways is mediated by cytoplasmic coat protein complexes associated with the cytosolic face of membranes (reviewed in Rothman and Wieland 1996; Schekman and Orci 1996). One class of well characterized coat protein complexes contains clathrin and adaptor protein (AP) complexes AP-1, AP-2, or AP-3 (reviewed in Kirchhausen et al. 1997; Hirst and Robinson 1998; Lewin and Mellman 1998). AP-1 is mainly localized to the *trans*-Golgi network (TGN) and mediates protein trafficking between the TGN and endosomes, AP-2 is localized to the plasma membrane and mediates receptor endocytosis (reviewed in Kirchhausen et al. 1997; Hirst and Robinson 1998; Lewin and Mellman 1998), while AP-3 is localized to endosomes and/or the TGN and, in mammals, is thought to mediate protein sorting to lysosomes and specialized endosomal-lysosomal organelles (Simpson et al. 1996, 1997; Dell'Angelica et al. 1997, 1998, 1999b; Kantheti et al. 1998; Le Borgne et al. 1998; Feng et al. 1999). AP complexes consist of a small chain (σ1, σ2, or σ3; 17–23 kDa), a medium chain (μ1, μ2, or μ3; 47–50 kDa) and two large chain adaptins: β1, β2, or β3 (100–140 kDa), and γ ($\approx$100 kDa), α ($\approx$100 kDa), or δ ($\approx$130 kDa) for AP-1, AP-2, or AP-3, respectively. Recently a fourth adaptor complex, AP-4, was identified in mammals and shown to consist of a small chain (σ4, $\approx$17 kDa), a medium chain (μ4, $\approx$50 kDa), and two large chain adaptins: β4 ($\approx$83 kDa) and ε ($\approx$140 kDa) (Dell'Angelica et al. 1999a).

Specific roles have been ascribed to individual AP subunits. The β1-, β2-, and β3-adaptins of AP-1, AP-2, and AP-3, respectively, interact with clathrin (Ahle and Ungewickell 1989; Gallusser and Kirchhausen 1993; Shih et al. 1995; Dell'Angelica et al. 1998) and with di-leucine-based sorting signals in the cytosolic tails of some integral membrane proteins (Greenberg et al. 1998; Rapoport et al. 1998). The γ- and α-adaptins are thought

to target AP-1 and AP-2 to the TGN and the plasma membrane, respectively (Robinson 1993; Page and Robinson 1995), and to bind regulatory molecules (Benmerah et al. 1996; Tebar et al. 1996; Schmid 1997; Chen et al. 1998). The μ1, μ2, and μ3 subunits bind specific tyrosine-based sorting signals (Ohno et al. 1995, 1996, 1998; Boll et al. 1996; Dell'Angelica et al. 1997; Rapoport et al. 1997; Stephens et al. 1997). Mapping of functional domains in μ1 and μ2 demonstrated a bipartite organization of these proteins: the N-terminal third is involved in the interaction with β-adaptins, while the remainder is responsible for binding tyrosine-based sorting signals (Aguilar et al. 1997). The function of the σ1, σ2, and σ3 subunits is unknown. The β, δ, and ζ chains of another coat protein complex, COPI, bear limited sequence similarity to the β, μ, and σ AP subunits, respectively (Duden et al. 1991; Kuge et al. 1993; Faulstich et al. 1996), suggesting they may play similar roles in vesicle formation and/or recognition of sorting motifs.

The compositions of AP complexes and COPI have been remarkably conserved in all eukaryotes examined to date, from yeast to humans. It is not clear, however, to what extent this conservation reflects similar functional roles in different organisms. For example, null mutations in AP-1 subunit genes in yeast cause no detectable phenotype (reviewed in Phan et al. 1994), whereas null mutations in the homologous genes in *Caenorhabditis elegans* (Lee et al. 1994) and mice (Zizioli et al. 1999) are embryonic lethal. Our laboratory has been studying adaptor function in metazoans using *Drosophila melanogaster* as a model system. Here, we present the sequences of the Drosophila AP medium (μ) chains μ1, μ2 and μ3, and δ-COP, the analogous subunit of the COPI coat complex. The genes encoding the medium chains and δ-COP were mapped in the Drosophila genome, permitting correlation with mutations that may affect protein sorting. The gene for the Drosophila μ3 chain was found to map to the *carmine* (*cm*) locus; the *cm* mutation affects pigmentation, a process known to require efficient protein trafficking (Ooi et al. 1997; Shestopal et al. 1997; Warner et al. 1998; reviewed by Lloyd et al. 1998). *cm* is one of a group of genetically interacting pigmentation mutations which includes the δ-adaptin mutant *garnet*, as well as other mutants in protein sorting (Lloyd et al. 1998). Analysis of genomic DNA from the cm^1 mutant indicated the presence of a large insertion in the coding region of the μ3 gene. Northern analysis of RNA from cm^1 flies revealed no detectable μ3 mRNA. In addition, light microscopy demonstrated a reduction in the number of pigment granules in the eyes of cm^1 flies. These findings suggest an important role for μ3 in protein trafficking pathways involved in pigment granule biogenesis, as well as providing the first description of a μ3 mutant in any multicellular organism.

Materials and methods

Identification of Drosophila cDNAs for adaptor medium chains and δ-COP

A sequence similarity search of the Berkeley Drosophila Genome Project (BDGP) EST database, using the BLAST algorithm (Altschul et al. 1990) and protein sequences corresponding to murine AP47 (μ1A) (Accession No. M62419), human AP50 (μ2) (Accession No. U36188), mouse p47A (μ3A) (Accession No. L07073), and human δ-COP (Accession No. X81198) identified a number of clones encoding polypeptides that are highly similar to their mammalian counterparts. ESTs encoding putative Drosophila μ1 (LD14502, Accession No. AA439826), μ2 (GM02287, Accession No. AA567297), μ3 (LD09732, Accession No. AA390968), and δ-COP (CK00375, Accession No. AA140670) proteins were obtained from Genome Systems (St. Louis, Mo.) and sequenced using an ABI Prism Model 377 sequencer (Perkin Elmer, Foster City, Calif.). DNA sequences were assembled and consensus sequences derived and translated using the AutoAssembler DNA analysis software, version 1.4 (Perkin Elmer). Sequence analysis revealed that the δ-COP EST (CK00375) was incomplete at its 5′ end. The sequence of the first 33 residues of Drosophila δ-COP was obtained from the known 5′ sequence of EST LD30910 (Accession No. AA950785) and was used in compiling the complete protein sequence presented here. While this work was in progress, the sequences of μ1, μ2, and μ3 were posted in GenBank by Y. Q. Zhang and K. S. Brodie.

Cytological mapping of genes for adaptor medium chains and δ-COP

The μ1, μ2, and δ-COP genes were mapped to the Drosophila genome by hybridization to a filter (Genome Systems) dotted with P1 DNA clones that cover the Drosophila genome and have been cytologically mapped by conventional in situ hybridization to salivary gland polytene chromosomes. cDNA inserts were isolated and radiolabeled probes were produced using the Megaprime DNA labeling system (Amersham, Arlington Heights, Ill.). Resulting signals were used to determine cytological positions of genes for medium chains and δ-COP with the aid of conversion tables supplied by the manufacturer and information provided on the BGDP web server (http://fruitfly.berkeley.org/). Attempts to map the Drosophila μ3 gene by this approach were unsuccessful. The cytological position of μ3 was obtained from the BDGP database (Accession No. 3341417; Y. Q. Zhang Y. Q. and K. S. Brodie, unpublished results).

Fly strains

The *D. melanogaster carmine* mutant (cm^1) and CantonS (wild type) strains were obtained from the Bloomington Stock Center (Bloomington, Ind.). *cardinal* (*cd*) mutants (cd^{kn50}, cd^{kn308}, and cd^{kn211}) were obtained from Dr. Eiji Nitasaka (Kyushu University, Fukuoka, Japan).

Microscopy and spectrophotometric analyses of Drosophila eye pigments

For morphological analyses, adult heads were dissected and fixed by overnight immersion in 2% glutaraldehyde in 100 mM HEPES buffer with 3% sucrose at pH 7.4. Fly heads were then bisected, post-fixed in 2% reduced osmium tetroxide in 100 mM sodium cacodylate buffer, and dehydrated through a graded series of ethanol solutions from 50% to 100%. Fly heads were then infiltrated overnight with a 1:1 mixture of Spurr resin (Electron Microscopy Sciences, Ft. Washington, Pa.) and propylene oxide, followed by three changes of 100% Spurr resin for 2 h each.

Tangential (*en face*) sections and longitudinal sections were cut from eyes obtained from five flies of each type at an approximate thickness of 0.5 μm using an ultramicrotome, and stained with 1% Toluidine Blue and 1% sodium borate. Sections were then viewed using transmitted light and photographed. Extraction and quantitation of the red (pteridine) and brown (ommochrome) pigments from adult eyes was performed as described previously (Ooi et al. 1997).

Northern and Southern analyses

To examine developmental expression patterns, total RNA was isolated from flies of the specified developmental stage (0–20 h embryos, wandering third-instar larvae, early stage light pupae, late stage dark pupae, and 3-day-old males or females) using Trizol (Life Technologies, Gaithersburg, Md.). RNA samples (15 μg) were fractionated on a 1.2% formaldehyde/agarose gel and transferred to Hybond N+ membrane (Amersham). Hybridization of μ1, μ2, μ3, or δ-COP probes was performed using conditions described for the P1 filter analysis. To examine μ3 expression in wild-type and cm^1 flies, total RNA was extracted from adults using Trizol. RNA samples (10 μg) were fractionated on a 1% formaldehyde/agarose gel and transferred to GeneScreen Plus membrane (New England Nuclear, Boston, Mass.). Hybridization was performed using the full-length μ3 cDNA sequence and a cDNA for the Drosophila TATA binding protein (TBP) was used as a loading control (Ooi et al. 1997). Hybridized filters were examined by autoradiography.

For Southern analyses, 5 μg of genomic DNA was digested with restriction endonucleases and resolved on 0.8% agarose gels. DNA was transferred to GeneScreen Plus membrane (New England Nuclear) and hybridized with radiolabeled probes: full-length Drosophila medium chain or δ-COP cDNAs (Fig. 3); the δ-adaptin cDNA, the μ3 cDNA, nucleotides 1–113 (5′ probe) of the μ3 ORF, or nucleotides 1090–1440 (3′ probe) of the μ3 ORF (Fig. 5).

Results

Identification of AP medium chain and δ-COP orthologs in Drosophila

To identify the genes for AP complex medium (μ) chains and δ-COP in Drosophila, a search of the expressed sequence tag (EST) database of BDGP was performed using mammalian μ1A, μ2, μ3A, and δ-COP protein sequences (see Materials and methods). This search yielded a number of ESTs encoding putative Drosophila medium chains. EST clones were sequenced and conceptual translation of the ORFs revealed medium chain proteins with the following predicted molecular weights: μ1, 48.9 kDa; μ2, 49.8 kDa; and μ3, 46.7 kDa (Fig. 1). These values are in good agreement with those for known medium chain family members from other organisms (Thurieau et al. 1988; Nakayama et al. 1991; Lee et al. 1994; Pevsner et al. 1994). ESTs encoding a Drosophila ortholog of mammalian δ-COP were also identified in this search. Drosophila δ-COP (Fig. 1) has a predicted molecular weight of 57.8 kDa, similar to mammalian δ-COP proteins (Radice et al. 1995; Faulstich et al. 1996).

Evolutionary conservation of Drosophila medium chain and δ-COP proteins

Comparisons of protein sequences revealed extensive sequence identity between Drosophila medium chains and δ-COP and their respective orthologs in mammals

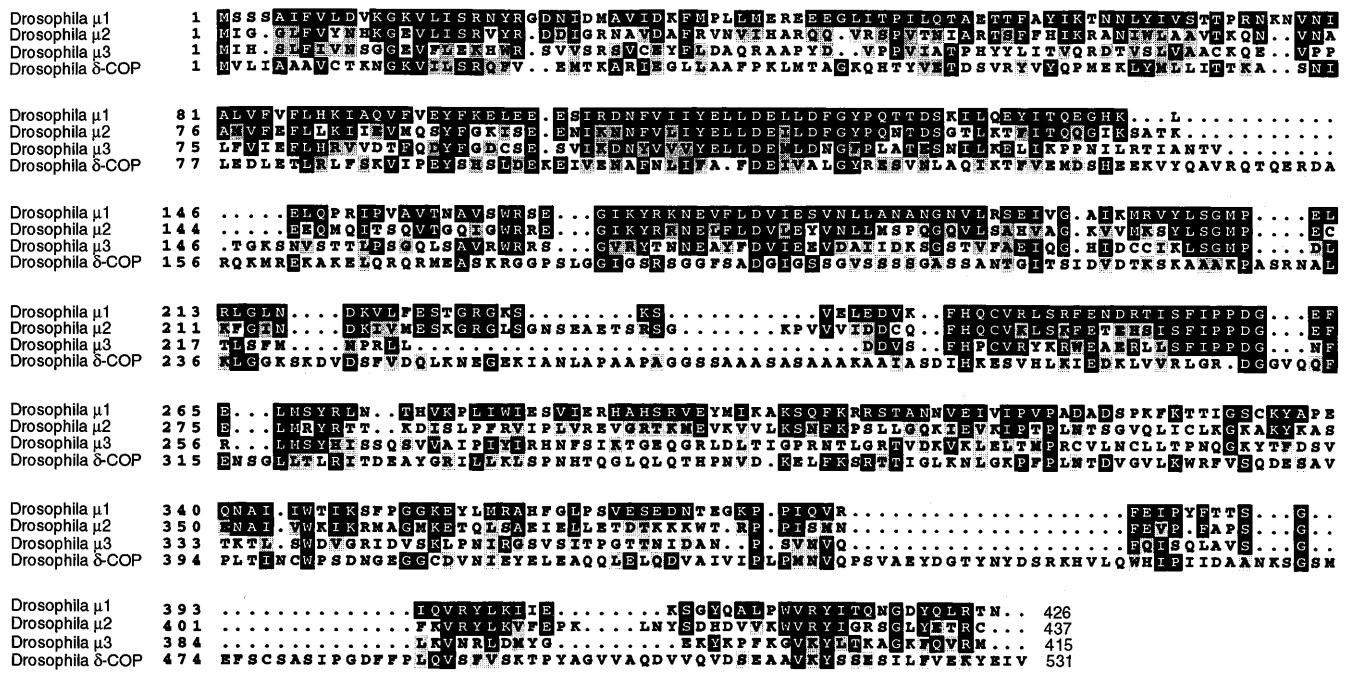

Fig. 1 Amino acid sequences of Drosophila adaptor medium chains and δ-COP proteins. Amino acid sequences of Drosophila medium chains μ1 (Accession No. AF110231), μ2 (Accession No. AF110232), μ3 (Accession No. AF110233), and δ-COP (Accession No. AF110234 for partial sequence) proteins are presented. Sequence identities (with reference to μ1) are shaded in *black* and similarities are shaded in *gray*. Alignments were produced using the ClustalW Multiple Sequence Alignment software (available at the European Bioinformatics Institute website http://www2.ebi.ac.uk/clustalw/) and shaded using the BOXSHADE program (available at the Pedro's BioMolecular Research Tools website http://www.biophys.uni-duesseldorf.de/bionet/rt_1.html)

and *C. elegans* (Table 1). Drosophila μ1, μ2, and μ3 displayed somewhat less identity to their respective *S. cerevisiae* orthologs APM1, APM4, and APM3 (Cowles et al. 1997; Panek et al. 1997) (Table 1). A similar lower level of identity is observed between Drosophila and yeast δ-COP proteins. The Drosophila μ2 chain bears some overall sequence identity to a C-terminal region of the protein STNB, another medium chain-type protein from Drosophila encoded by the *stonedB* gene (Andrews et al. 1996).

Phylogenetic analysis reveals that the Drosophila medium chains and δ-COP protein tightly cluster with their respective orthologs (Fig. 2). Members of the medium chain family diverged from δ-COP proteins at an early stage. Less evolutionary conservation is seen between the Drosophila and yeast medium chain orthologs. Because mammalian μ3A and μ3B are isoforms they cluster closer to each other than to other μ3 chains. The Drosophila STNB protein is much less related to the medium chains than μ1, μ2, or μ3 orthologs are to each other, suggesting that STNB, though more similar to the μ-chains than to δ-COP proteins, is only loosely related to the medium chains.

To determine if the medium chains and δ-COP proteins are each encoded by a single genomic transcription unit, Drosophila genomic DNA was digested with restriction endonucleases and subjected to Southern analysis. Blots were hybridized with radiolabeled probes corresponding to full-length μ1, μ2, μ3, and δ-COP cDNA sequences (see Materials and methods) (Fig. 3). Under the stringency conditions used, only one hybridization signal is visible in each lane for each of the four genes examined. This strongly suggests that each of the Drosophila proteins μ1, μ2, μ3, and δ-COP is encoded by a single transcription unit.

Expression of medium chain and δ-COP genes during Drosophila development

To examine the developmental expression pattern of the medium chains and δ-COP, total RNA was isolated from flies at various stages of development and subjected to Northern analysis using probes for μ1, μ2, μ3, and δ-COP (see Materials and methods). Medium chain and δ-COP transcripts were detected throughout development, from the embryo to the adult (Fig. 4). Similar findings were obtained for μ1 and μ2 upon examination of embryos at 0–1.5, 1.5–3.0, 3.0–4.5, 4.5–6.0, 6.0–7.5 and 7.5–24.0 h (data not shown).

Cytological mapping of Drosophila medium chain and δ-COP genes

A number of studies have demonstrated that sorting of proteins to pigment granules in Drosophila is mediated by many of the same proteins required for trafficking to endosomes and lysosomes (Ooi et al. 1997; Shestopal et al. 1997; Warner et al. 1998). Mutations in some components of this sorting machinery lead to defects in pigment granules, thus producing a range of mutant eye-color phenotypes. To determine if the Drosophila medium chain or δ-COP genes map to positions of identified Drosophila eye-color mutations, a high-density P1 filter was hybridized with probes corresponding to μ1, μ2, μ3, and δ-COP cDNAs (see Materials and methods). Cytological locations, as well as P1 clones and contigs, to which these genes were localized, are presented in Table 2. This approach was, however, unsuccessful in the case of μ3.

The map positions of the medium chain and δ-COP genes were then examined for eye color mutations, using information available on the FlyBase website (http://flybase.bio.indiana.edu/). This analysis revealed that the μ3 gene, which maps to cytological position 6E on the *X* chromosome (Y. Q. Zhang and K. S. Brodie, unpublished results), localizes to a region that includes the *carmine* (*cm*) mutation (Mohr 1927), which maps to position 6E6–6E7. Phenotypically, *cm* flies display abnormal pigmentation relative to wild-type flies (Ferre et al. 1986).

Analysis of μ3 expression in the cm^1 mutant

To examine the μ3 gene in *cm* mutants, genomic DNA was isolated from wild-type and cm^1 flies, digested with restriction endonucleases, and subjected to Southern analysis, using radiolabeled probes corresponding to the full-length or the 3′ region of the μ3 cDNA (see Materials and methods). The full-length μ3 probe detected two *Bam*HI fragments of approximately 7 kb and 1.3 kb (Fig. 5A, lane 1) and a single *Eco*RI fragment of approximately 10 kb (lane 2) in wild-type flies. In contrast, in the cm^1 mutant three *Bam*HI fragments of approxi-

Table 1 Amino acid sequence comparisons

Protein	Reference protein/percentage similarity[a]			
Drosophila μ1	Mouse μ1A/81	*C. elegans* μ1/75	*S. cerevisiae* APM1/52	
Drosophila μ2	Mouse μ2/87	*C. elegans* μ2/82	*S. cerevisiae* APM4/32	Drosophila STNB/13[b]
Drosophila μ3	Mouse μ3A/68	*C. elegans* μ3/66	*S. cerevisiae* APM3/17	
Drosophila δ-COP	Human δ-COP/59	*S. cerevisiae* δ-COP/26		

[a] See the legend to Fig. 2 for GenBank accession numbers
[b] Residues 661-1221 were used for the comparison

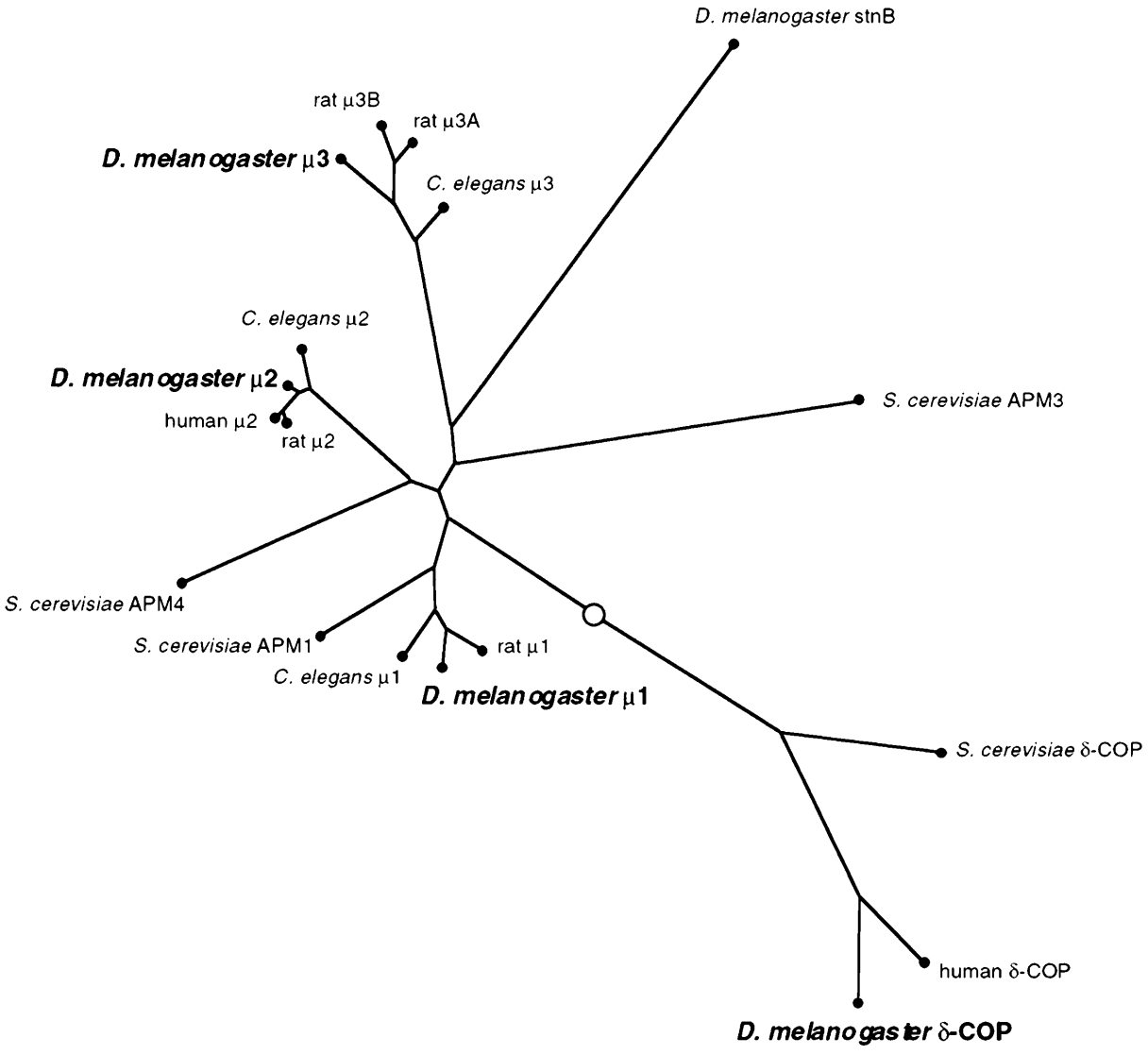

Fig. 2 Phylogenetic analysis of adaptor medium chains and δ-COP proteins. The phylogenetic tree compares the sequences of Drosophila μ1, μ2, μ3, and δ-COP proteins with medium chain and δ-COP proteins from mammals, *C. elegans*, and the yeast *S. cerevisiae*; and the STNB protein from Drosophila. The tree was constructed using the Darwin program (Gonnet 1992). The length of each branch represents the phylogenetic distance in PAM units (accepted point mutations for every 100 amino acid residues). The position of the putative ancestral precursor to the medium chains and δ-COP proteins is represented by the *empty circle*. GenBank Accession Nos. are as follows: mouse μ1, M62419; *C. elegans* μ1 (Unc-101), L26291; human μ2, U36188; *C. elegans* μ2 (CEAP50), 543818; *S. cerevisiae* APM1, 1703330; mouse μ3A (p47A), L07073; mouse μ3B (p47B), L07074; *S. cerevisiae* APM2, 2492680; *C. elegans* μ3, 1086692; *D. melanogaster* STNB, U54982; human δ-COP, 1351970; *S. cerevisiae* δ-COP, 1176026; *S. cerevisiae* APM3, 586357

mately 11, 1.4 and 1.3 kb (lane 4) and two *Eco*RI fragments of approximately 12 and 3.5 kb (lane 5) were observed. This suggested the presence of an aberration at the μ3 locus in the cm^1 mutant. Analyses using a probe from the 3′ terminus of the μ3 ORF (lanes 7–12) were performed to identify and orient the bands observed in wild-type and cm^1 flies. This probe hybridized only to the 7- and 10-kb fragments (lanes 7–9) in wild-type flies and the 11 and 12 kb fragments (lanes 10–12) in cm^1 flies. Examination of the cm^1 mutant using a 5′ terminal μ3 probe revealed only the 3.5-kb *Eco*RI and 1.3-kb *Bam*HI fragments (data not shown). In combination, these results predict that a large insertion (≈5 kb) is present 3′ to the *Bam*HI site in the μ3 ORF (Fig. 5B). This insertion is predicted to contain one *Bam*HI and one *Eco*RI site in its 5′ region; the distance between the *Bam*HI sites in the μ3 sequence and insertion is approximately 1.4 kb. A probe corresponding to full length δ-adaptin was also used to assess the efficiency of endonuclease digestion and loading (Fig. 5A, lanes 13–18).

The ≈5 kb insertion in the μ3 gene locus would be predicted to produce a μ3 mRNA of approximately 6.5 kb or, alternatively, lead to an unstable mRNA. To analyze the μ3 mRNA, total RNA from wild-type and homozygous cm^1mutant flies was subjected to Northern analysis using a radiolabeled probe comprising the full-length μ3 cDNA sequence (see Materials and methods).

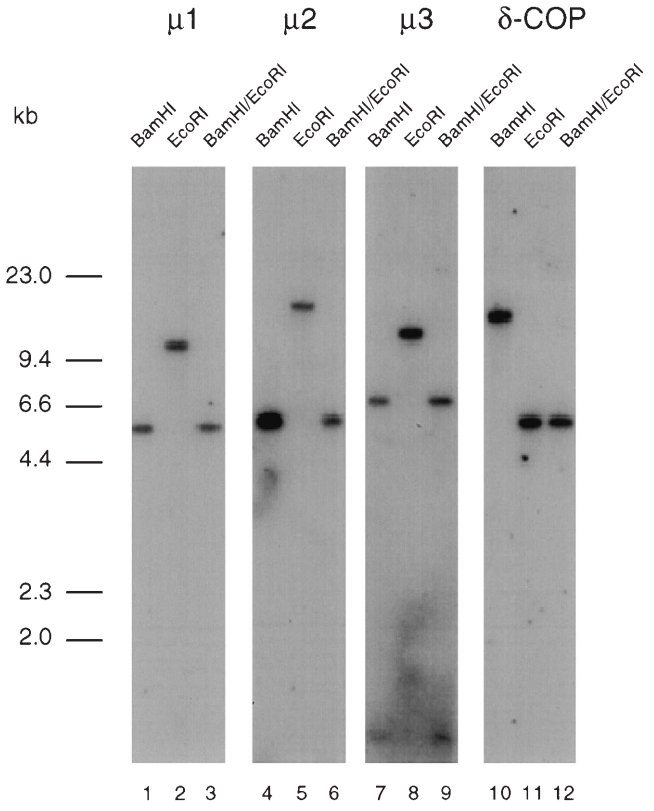

Fig. 3 Southern analysis of Drosophila genomic DNA. Genomic DNA was isolated from CantonS (wild-type) flies and digested with the indicated restriction endonucleases. Filters were hybridized with probes corresponding to full-length cDNA sequences for Drosophila μ1 (lanes 1–3), μ2 (lanes 4–5), μ3 (lanes 7–9), and δ-COP (lanes 10–12). Relative positions of DNA size markers (in kb) are indicated on the left

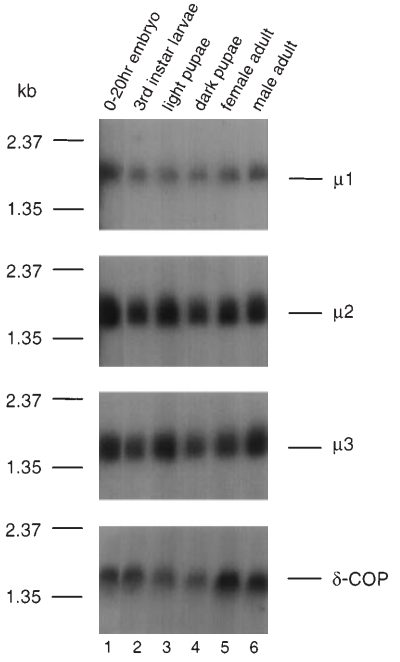

Fig. 4 Expression of adaptor medium chains and δ-COP during Drosophila development. Northern blots of RNA from various Drosophila developmental stages were analyzed with radiolabeled probes specific for Drosophila μ1, μ2, μ3, and δ-COP cDNA sequences (see Materials and methods). The positions of RNA size markers (in kb) and medium chain and δ-COP mRNAs are indicated

No μ3 mRNA was detected in the cm^1 flies (Fig. 6, lane 2) relative to wild type (lane 1), suggesting that the 3′ insertion mutation leads to an unstable μ3 transcript. A similar finding was recently reported for the mouse pigmentation mutant *pearl* in which a duplication within the β3A gene results in an unstable β3A mRNA (Feng et al. 1999).

The μ2 gene mapped to a position (94B1–94B2) that falls within the cytological region (94A1–94E2) known to contain the *cardinal* (*cd*) pigmentation mutation. However, Northern analysis revealed a μ2 transcript of apparently normal size and abundance in flies heterozygous for *cd* alleles cd^{kn50}, cd^{kn308}, and cd^{kn211} (data not shown). Future studies of μ2 transcript level and sequence in homozygous *cd* flies will be required to definitively elucidate the possible relationship between the *cd* locus and the μ2 gene.

Analysis of pigment granules in wild-type and *carmine* mutant flies

The compound eye of Drosophila is composed of 700–800 units termed ommatidia. At its apical end, each ommatidium contains a corneal lens through which light is focused onto seven photoreceptor cells, which run the length of the ommatidium (for a detailed description of Drosophila eye structure, see Cagan and Ready 1989). At the apical surface of each ommatidium primary pigment cells are found, which contain primary pigment granules that house ommochrome (brown) pigments. Along the central face of each photoreceptor cell is a photosensitive rhabdomere (R). The apical two-thirds of an ommatidium contains rhabdomeres R1–R7, while R7 is replaced by R8 in the remaining one third. In addition, ommatidia are optically separated from each other by a sheath of secondary and tertiary pigment cells containing secondary and tertiary pigment granules, respectively; these house both ommochrome and pteridine (red) pigments.

To assess levels of eye pigmentation, wild-type and cm^1 mutant flies were analyzed by light microscopy (Fig. 7). On cursory examination, cm^1 eyes (Fig. 7B) are phenotypically darker than *Canton S* (wild-type) eyes (Fig. 7A). Tangential sections of eyes (see Materials and methods) revealed a dramatic reduction in the number of secondary and tertiary pigment granules surrounding each ommatidium in cm^1 (Fig. 7D) relative to wild-type flies (Fig. 7C). This analysis was performed at multiple levels from the apical (R7 level) to the distal (R8 level) end of the ommatidia; all the results were consistent with the sections shown in Fig. 7C, D). Longitudinal sections that include primary granules located in the primary pigment cells at the apical end of each ommatidium

Table 2 Locations of genes encoding AP medium (μ) chains and δ-COP protein in the Drosophila genome

Gene	Cytological position[a]	P1 clones	Contig
μ1	85D20–85D23	DS01769, DS01540	DS04945
μ2	94B1–94B2	DS01767, DS02968, DS04104, DS02329, DS03730, DS06822	DS01102
μ3[b]	6E	–	–
δ-COP	2A1–2B18	DS06463, DS02478	Arm

[a] Cytological positions of μ1, μ2, and δ-COP genes were derived from map information available from the Berkeley Drosophila Genome Project (BDGP) for contig DS04945, contig DS01102, and P1 clones DS06463 and DS02478, respectively

[b] Sequences for Drosophila AP47 (μ1) (Accession No. AJ006219), AP50 (μ2) (Accession No. AJ005962), and μ3 (Accession No. 3341417) have also recently been entered in the database and mapped to regions 85D, 94B, and 6E, respectively, in the Drosophila genome (Y. Q. Zhang and K. S. Brodie, unpublished results). The map position of μ3 presented here was obtained from the database

(Fig. 7E, F) and secondary and tertiary granules found in subretinal cells positioned at the basal end of each ommatidium (Fig. 7G, H), reveal a reduction in the numbers of granules in *cm*^{1} relative to wild-type flies. In addition, we have observed ommatidia that lack an outer rhabdomere in *cm*^{1} flies (see arrow in Fig. 7D). This initially suggested a possible defect in eye development in these flies. However, further scoring of ommatidia revealed no significant difference in rhabdomere number between *cm*^{1} and wild-type flies (data not shown).

To obtain a more quantitative estimation of eye pigmentation, pteridine and ommochrome pigments were extracted from the eyes of wild-type and *cm*^{1} flies (see Materials and methods). The absorption spectra of the pigments were then measured throughout the visible

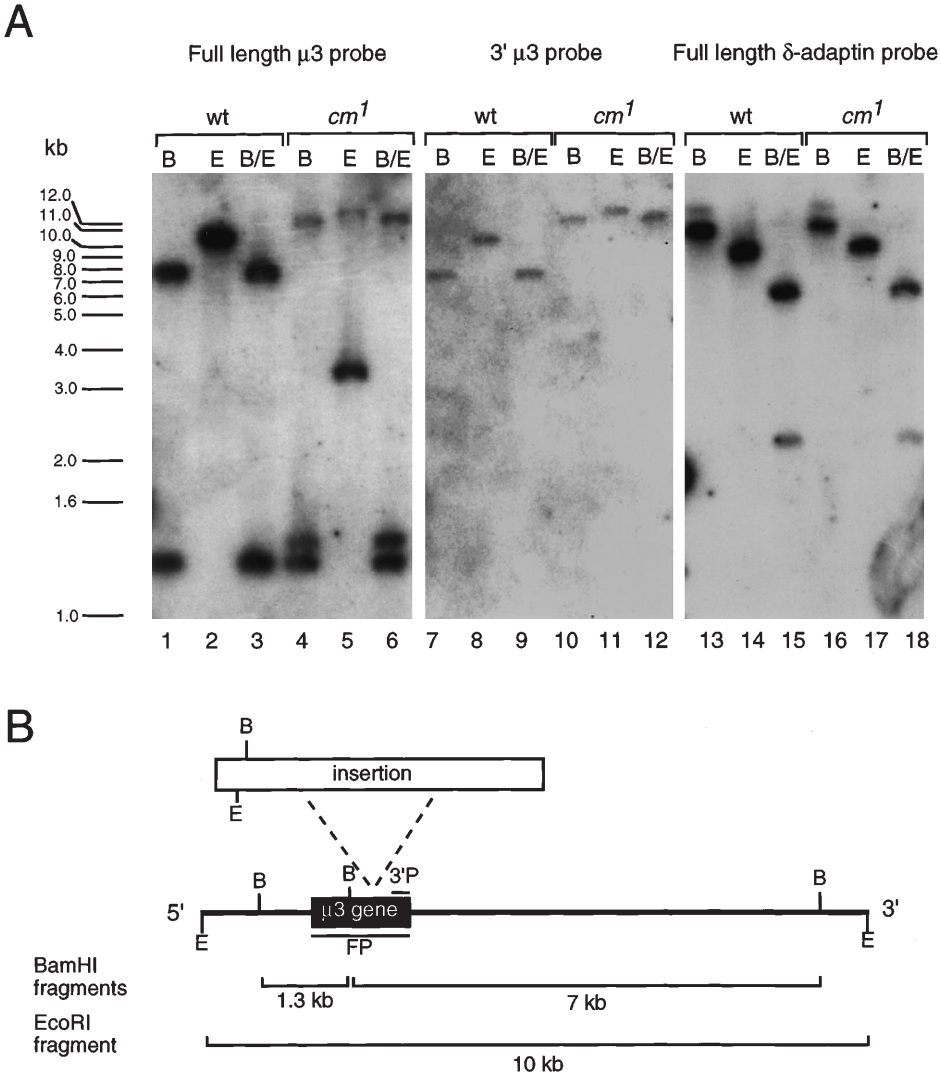

Fig. 5A, B Analysis of the μ3 gene. **A** Southern analysis was performed on genomic DNA from CantonS (wt) and *cm*^{1} mutant flies digested with *Bam*HI (B), *Eco*RI (E), or *Bam*HI and *Eco*RI (B/E). Radiolabeled probes were used corresponding to the full-length μ3 coding sequence (lanes 1–6), a 3′ terminal sequence (lanes 7–12) of μ3, and the full-length δ-adaptin coding sequence (lanes 13–18) (see Materials and methods). The mobility of DNA size markers is indicated on the left. **B** Schematic diagram of the μ3 locus. The μ3 gene and the insertion sequence found in the *cm*^{1} mutant are represented by the *black box* and the *open box*, respectively. Relative positions of *Bam*HI (B) and *Eco*RI (E) sites are indicated based on the data in **A**. *Bam*HI and *Eco*RI fragment sizes are based on the restriction pattern of the wild-type sequence. Locations of sequences corresponding to the 3′ terminal (3′P) and full length (FP) μ3 probes are indicated. The Figure is drawn roughly to scale

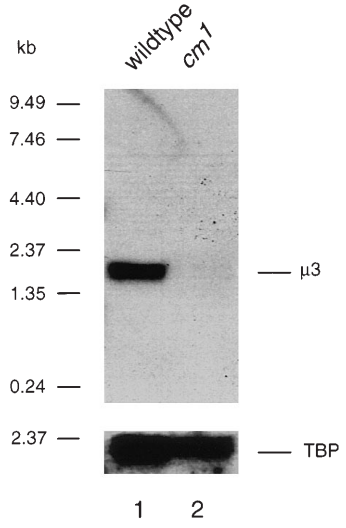

Fig. 6 Expression of $\mu 3$ mRNA in wild-type and cm^1 mutant flies. Total RNA was extracted from CantonS (wild-type) (lane 1) and cm^1 mutant flies (lane 2). The blot was hybridized with a probe representing the full-length Drosophila $\mu 3$ cDNA sequence. As a control for RNA loading, the blot was hybridized with a probe corresponding to the Drosophila TBP gene. Relative positions of RNA size markers (in kb) are indicated on the left

range (Fig. 8). This analysis revealed a reduction in levels of both pteridine and ommochrome pigments in the cm^1 mutant relative to the wild-type control, consistent with similar studies performed by Nolte (1950). Thus, this quantitative reduction in the abundance of both red and brown pigments correlates with the reduction in pigment granule numbers observed in cm^1 eyes by light microscopy.

Discussion

In this study we report the identification of four members of the adaptor medium chain family, namely $\mu 1$, $\mu 2$, and $\mu 3$, and δ-COP in Drosophila. These proteins display a high level of identity to their metazoan counterparts and somewhat less identity to the corresponding yeast proteins (Table 1) and form a tight cluster with their respective orthologs in phylogenetic analyses (Fig. 2). A high degree of evolutionary conservation is also seen for other Drosophila AP subunits, such as α-adaptin of AP-2 (Dornan et al. 1997; Gonzalez-Gaitan et al. 1997) and δ-adaptin of AP-3 (Ooi et al. 1997). These observations suggest strong evolutionary pressure to preserve the specialized functions of $\mu 1$, $\mu 2$, and $\mu 3$, and δ-COP and of the complexes to which they belong (i.e. AP-1, AP-2, AP-3 and COPI). Interestingly, $\mu 4$, the medium chain of the novel adaptor complex AP-4 (Dell'Angelica et al. 1999a), does not appear to be present in Drosophila.

Moreover, we have identified a mutation in the $\mu 3$ gene in the eye-color mutant *carmine* which results in a lack of detectable $\mu 3$ mRNA. Light microscopy studies of the *cm* mutant reveals a reduction in pigment granule numbers in the adult eye, demonstrating a role for $\mu 3$ in the sorting processes required for pigment granule biogenesis in Drosophila.

A well known function of mammalian $\mu 1$, $\mu 2$, and $\mu 3$ chains is the recognition of tyrosine-based sorting signals conforming to the YXXØ motif (where Y is tyrosine, X is any amino acid and Ø is leucine, isoleucine, phenylalanine, methionine or valine) (Ohno et al. 1995, 1996, 1998; Boll et al. 1996; Dell'Angelica et al. 1997; Rapoport et al. 1997; Stephens et al. 1997; reviewed by Bonifacino and Dell'Angelica 1999). Tyrosine-based sorting signals serve to target integral membrane proteins for endocytosis from the cell surface and to the lysosome. Recent X-ray crystallographic studies have identified the precise residues in mammalian $\mu 2$ that interact with tyrosine-based signals conforming to the consensus motif YXXØ (Owen and Evans 1998). These residues, and most of the corresponding residues in $\mu 1$ and $\mu 3$, are conserved in Drosophila (Table 3), suggesting that Drosophila medium chains may also be able to interact with YXXØ-type signals with the same specificity as mammalian medium chains. Further studies will be required to address the question of whether the role of YXXØ-μ chain interactions in protein sorting, which is well documented in mammals, also extends to Drosophila and other metazoans.

Limited homology is observed between the C-terminal domains of the Drosophila $\mu 2$ chain and the STNB protein (Table 1). As the N-terminal third of μ-chains mediates assembly with β-adaptins while the remainder of the proteins contain the residues which bind tyrosine signals (Aguilar et al. 1997; Owen and Evans 1998), a similarity to C-terminal sequences of the medium chains may suggest that STNB functions in binding some type of sorting signal, though not as a subunit of an AP complex.

Analysis of the developmental expression pattern of the genes encoding $\mu 1$, $\mu 2$, $\mu 3$ and δ-COP in Drosophila revealed that these genes are expressed early (0–3 h embryo) and at sustained levels from embryogenesis, through the larval and pupal stages, and into adulthood. The gene encoding the α-adaptin subunit of AP-2 in Drosophila has also been shown to be expressed at all stages of development (Dornan et al. 1997). These observations suggest a requirement for components of clathrin-associated and non-clathrin-associated protein coats for development in multicellular organisms.

Mutations in the α-adaptin gene in Drosophila have been shown to result in embryonic lethality due to inhibition of endocytosis and synaptic vesicle recycling at presynaptic termini (Dornan et al. 1997; Gonzalez-Gaitan et al. 1997). To assess the role of adaptor μ subunits in Drosophila, we established the cytological locations of the corresponding genes and compared these locations to those of mutant loci that confer either neurological or pigmentation defects, phenotypes that have been linked to abnormal protein trafficking (Wu and Bellen 1997; Lloyd et al. 1998). We were unable to

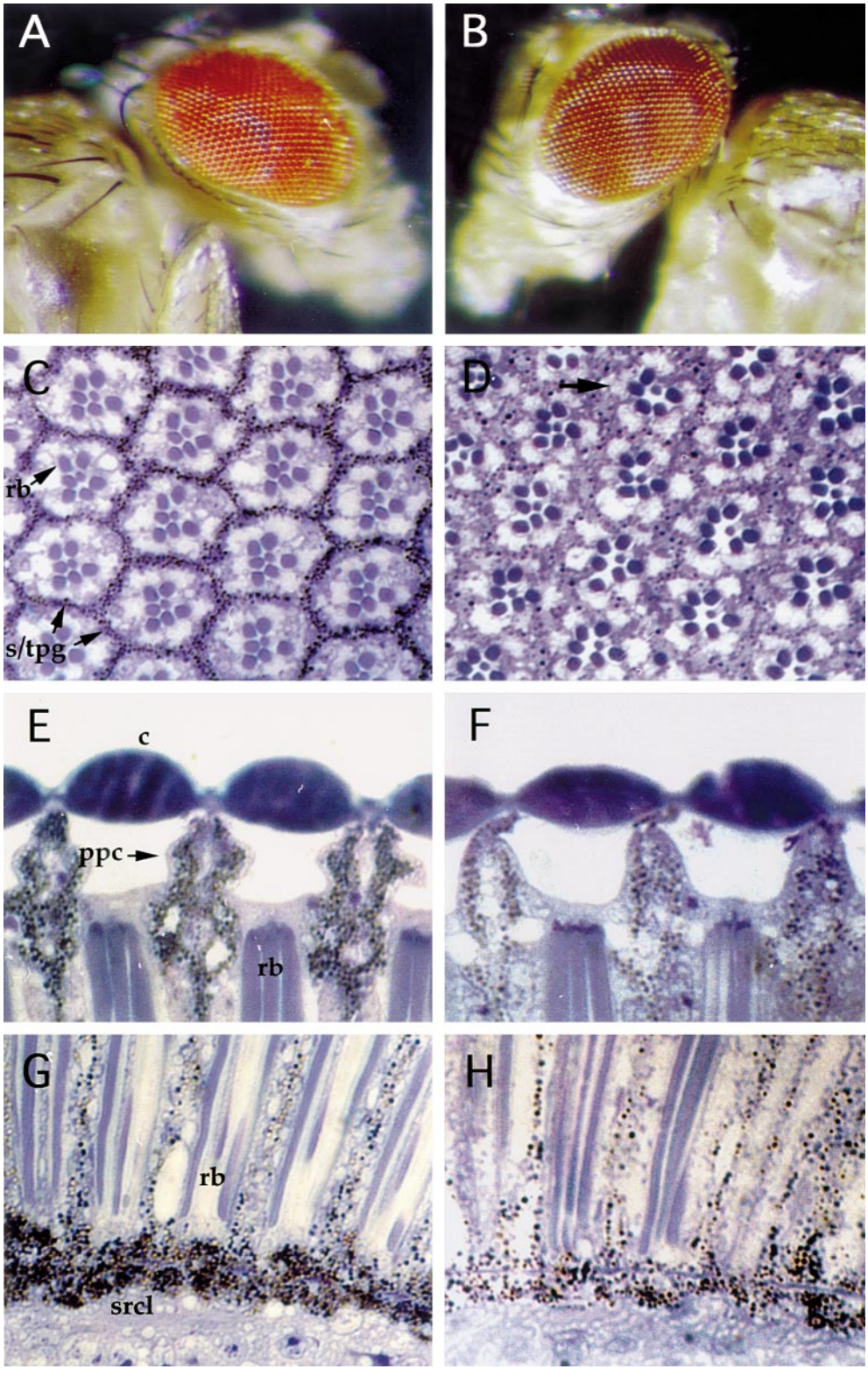

Fig. 7A–H Analysis of pigment granules in eyes of wild-type and cm^1 mutant flies by light microscopy. Light photomicrographs of CantonS (wild-type) and cm^1 mutant eyes are shown in panels **A** and **B**, respectively. **C**, **D** Light photomicrographs of 0.5-μm sections depicting a tangential (*en face*) view of CantonS (**C**) and cm^1 mutant (**D**) eyes at the R7 level. **E**, **F** Enlarged view of the primary pigment cells from CantonS (**E**) and cm^1 mutant (**F**) eyes. **G**, **H** Enlarged view of the subretinal pigment cell layer from CantonS (**G**) and cm^1 mutant (**H**) eyes. Positions of secondary and tertiary pigment granules (s/tpg), primary pigment cells (ppc), cornea (c), rhabdomere (rb), and subretinal cell layer (srcl) are indicated. An ommatidium containing six rhabdomeres is indicated by the *arrow* in panel **D**

link mutations in μ subunit genes to existing Drosophila neurological mutants, which led us to direct our search toward pigmentation mutants. Mutations in more than 80 genetic loci have been found to cause pigmentation defects in the eyes and other pigmented tissues of Drosophila (Phillips and Forest 1980; Lindsley and Zimm 1992). Detailed phenotypic characterization of these pigmentation mutants has allowed their classification into three different groups (Lloyd et al. 1998). The first group corresponds to mutations in genes encoding enzymes involved in the biosynthesis of either pteridine or ommochrome pigments (e.g. sepiapterin

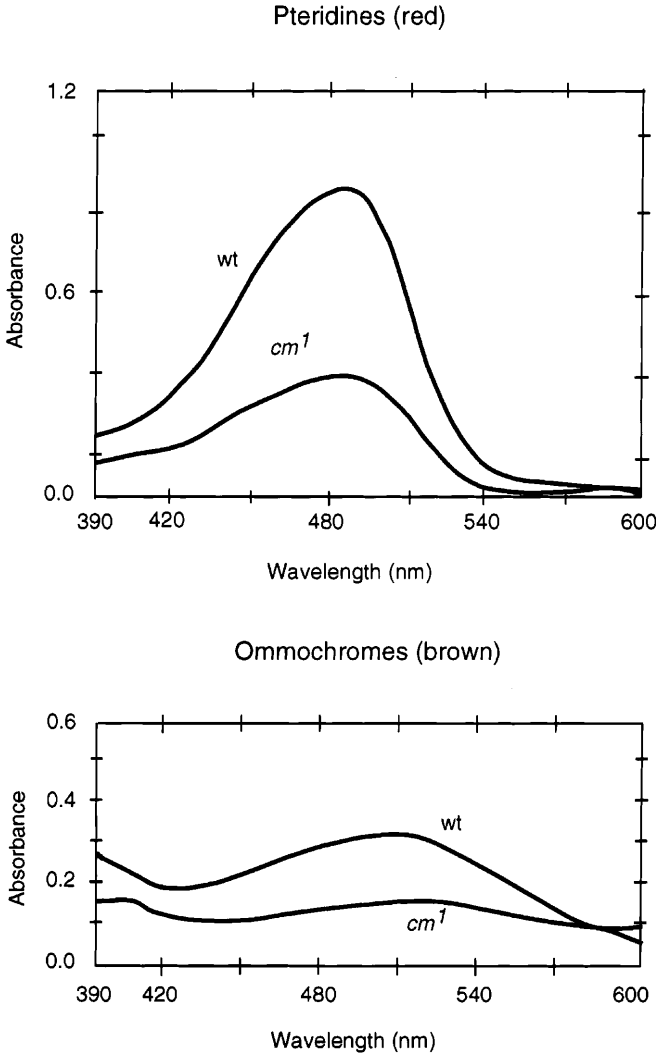

Fig. 8 Quantitation of red and brown pigments from wild-type and *cm*^{1} mutant flies. Pteridine (red) and ommochrome (brown) pigments were extracted from the eyes of CantonS (wild-type) and *cm*^{1} mutant flies. Pigments were then quantitated by measuring their absorption spectra in the 390–600 nm range

synthetase A and tryptophan oxygenase for the *purple* and *vermilion* mutations, respectively). Since these two pigments are made by different biosynthetic pathways, each mutation generally affects only one type of pigment. The second group includes mutants with defective expression of ABC membrane transporters involved in import of biosynthetic pigment precursors into pigment granules. Mutations in these genes affect either one (e.g. *scarlet*, *brown*) or both types of pigment (e.g. *white*). Finally, the third group (referred to as the "granule group", Lloyd et al. 1998) corresponds to mutations in the protein trafficking machinery. These mutations probably impair the targeting of biosynthetic enzymes to the pigment granules or the packaging of the pigments, and therefore tend to reduce both types of pigment. The first mutant of the granule group to be linked to a component of the sorting machinery was *garnet* (Ooi et al. 1997; Simpson et al. 1997) which bears a defect in the δ-adaptin subunit of AP-3. Other granule group mutants include *deep orange* and *light*, which encode homologs of the yeast vacuolar protein sorting genes *VPS18* and *VPS41*, respectively (Shestopal et al. 1997, Warner et al. 1998). In addition to their similar phenotypic characteristics, many granule group mutants display genetic interactions among themselves (Lloyd et al. 1998), suggesting that the mutations affect the same or alternative trafficking pathways required for pigment granule biogenesis.

Here we report the localization of the μ3 gene to a region of the *X* chromosome containing the granule group locus *carmine*. In flies homozygous for the mutant allele *cm*^{1}, we observed a $\approx$5 kb insertion into the μ3 gene and an apparent lack of μ3 mRNA. Relative to wild-type flies, *cm*^{1} flies have darker eyes and a dramatic reduction in the number of pigment granules in various types of pigment cells in the eye (Fig. 7). The *cm*^{1} mutant exhibited a depletion of both pteridine and ommochrome pigments, a characteristic of granule group mutants (Fig. 8). That a reduction in the number of pigment granules and pigment content should result in darker eye coloration may appear paradoxical. However, it has been shown that, when red and brown pigments are present in reduced amounts, the accumulation of "yellow" pigments, and their subsequent oxidation, can lead to phenotypically darker eyes in mutant flies (Ferre et al. 1986).

The consequences of the mutation in the μ3 gene for pigment granule biogenesis are similar to those reported previously for the δ-adaptin gene in Drosophila (Ooi et al. 1997). Since both μ3 and δ-adaptin are components of the AP-3 complex, these observations establish a critical role for this complex in pigment granule biogenesis in Drosophila. Remarkably, this role appears to have been conserved throughout evolution since mutations in the δ-adaptin gene in the mouse coat color mutant strain *mocha* (Kantheti et al. 1998), and of β3A-adaptin in the mouse coat color mutant strain *pearl* (Feng et al. 1999), and in two human patients with Hermansky-Pudlak syndrome (Dell'Angelica et al. 1999b), similarly result in reduced pigmentation. In addition to the pigmentation defects, mice and humans with altered expression of AP-3 subunits exhibit absence

Table 3 Comparison of residues potentially involved in binding YXXØ signals

Residue no. in Rat μ2	173–176	203	401	404	420–423
Rat μ2[a]	LFLD	K	V	L	KWVR
Drosophila μ2	LFLD	K	V	L	KWVR
Human μ2	LFLD	K	V	L	KWVR
Drosophila μ1	VFLD	R	V	L	PWVR
Mouse μ1A	VFLD	R	V	L	RWVR
Drosophila μ3	AYFD	C	V	L	KGVK
Rat μ3A	AYFD	C	V	L	KGVK

[a] Residues of the rat μ2 protein which interact with tyrosine-based signals (Owen and Evans 1998). Corresponding residues in Drosophila μ1, μ2, and μ3 and mouse μ1A and rat μ3A were identified based on sequence alignments (Owen and Evans 1998; this study)

of platelet dense granules and abnormalities in lysosomes of reticuloendothelial cells (Kantheti et al. 1998; Dell'Angelica et al. 1999b; Feng et al. 1999). All of these organelles are biogenetically related to lysosomes, suggesting that the underlying defect in AP-3 mutants is impaired protein transport to lysosome-related organelles. In agreement with this proposal, abnormal sorting of lysosomal/vacuolar membrane proteins has been demonstrated in AP-3-deficient yeast (Cowles et al. 1997; Stepp et al. 1997; Darsow et al. 1998; Vowels and Payne 1998) and mammalian cells (Le Borgne et al. 1998; Dell'Angelica et al. 1999b). Therefore, a likely explanation for the pigmentation defect observed in the *cm* flies is that biosynthetic enzymes, ABC transporters, or other proteins required for the production of pigments are not properly targeted to the site of their function in the absence of a complete AP-3 complex. Interestingly, the apparent null mutation in the cm^1 allele does not result in lethality. This is possibly due to redundancy in the sorting machinery requiring AP-3 function in Drosophila.

The results presented here thus provide further evidence that members of the granule group of pigmentation mutants bear mutations in components of the endosomal-lysosomal targeting machinery. Identification of the genes defective in other granule group mutants (e.g., *orange*, *ruby*, *claret*, *pink*, *purpleoid*, *lightoid*, Lloyd et al. 1998) may contribute to a detailed molecular description of this machinery in multicellular organisms, as well as provide additional candidate genes for analyses of pigmentation disorders in humans.

Acknowledgements The authors thank Eiji Nitasaka (Kyushu University, Japan) for *cardinal* stocks, Quang Phan for assistance in DNA sequencing, and Esteban Dell'Angelica for assistance in the generation and analysis of phylogenetic trees. We also thank Mary Lilly for critical reading of the manuscript. C.M. is supported by a Post-Doctoral Associateship from the National Research Council.

References

Aguilar RC, Ohno H, Roche KC, Bonifacino JS (1997) Functional domain mapping of the clathrin-associated adaptor medium chains μ1 and μ2. J Biol Chem 272:2760–2766

Ahle S, Ungewickell E (1989) Identification of a clathrin binding subunit in the HA2 adaptor protein complex. J Biol Chem 264:20089–20093

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Andrews J, Smith M, Merakovsky J, Coulson M, Hannan F, Kelly LE (1996) The *stoned* locus of *Drosophila melanogaster* produces a dicistronic transcript and encodes two distinct polypeptides. Genetics 143:1699–1711

Benmerah A, Begue B, Dautry-Varsat A, Cerf-Bensussan N (1996) The ear of alpha-adaptin interacts with the COOH-terminal domain of the Eps 15 protein. J Biol Chem 271:12111–12116

Boll W, Ohno H, Songyang Z, Rapoport I, Cantley LC, Bonifacino JS, Kirchhausen T (1996) Sequence requirements for the recognition of tyrosine-based endocytic signals by clathrin AP-2 complexes. EMBO J 15:5789–5795

Bonifacino JS, Dell'Angelica EC (1999) Molecular bases for the recognition of tyrosine-based sorting signals. J Cell Biol 145:923–926

Cagan RL, Ready DF (1989) The emergence of order in the Drosophila pupal retina. Dev Biol 136:346–362

Chen H, Fre S, Slepnev VI, Capua MR, Takei K, Butler MH, Di Fiore PP, De Camilli PD (1998) Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. Nature 394:793–797

Cowles CR, Odorizzi G, Payne GS, Emr SD (1997) The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. Cell 91:109–118

Darsow T, Burd CG, Emr SD (1998) Acidic di-leucine motif essential for AP-3-dependent sorting and restriction of the functional specificity of the Vam3p vacuolar t-SNARE. J Cell Biol 142:913–922

Dell'Angelica EC, Ohno H, Ooi CE, Rabinovich E, Roche KW, Bonifacino JS (1997) AP-3: an adaptor-like protein complex with ubiquitous expression. EMBO J 16:917–928

Dell'Angelica EC, Klumperman J, Stoorvogel W, Bonifacino JS (1998) Association of the AP-3 adaptor complex with clathrin. Science 280:431–434

Dell'Angelica EC, Mullins C, Bonifacino JS (1999a) AP-4, a novel protein complex related to clathrin adaptors. J Biol Chem 274:7278–7285

Dell'Angelica EC, Shotelersuk V, Aguilar RC, Gahl WA, Bonifacino JS (1999b) Altered trafficking of lysosomal membrane proteins in Hermansky-Pudlak syndrome due to mutations in the β3A subunit of the AP-3 adaptor complex. Mol Cell 3:11–21

Dornan S, Jackson AP, Gay NJ (1997) Alpha-adaptin, a marker for endocytosis, is expressed in complex patterns during Drosophila development. Mol Biol Cell 8:1391–1403

Duden R, Griffiths G, Frank R, Argos P, Kreis TE (1991) Beta-COP, a 110 kd protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to beta-adaptin. Cell 64:649–665

Faulstich D, Auerbach S, Orci L, Ravazzola M, Wegchingel S, Lottspeich F, Stenbeck G, Harter C, Wieland FT, Tschochner H (1996) Architecture of coatomer: molecular characterization of delta-COP and protein interactions within the complex. J Cell Biol 135:53–61

Feng L, Seymour AB, Jiang S, To A, Peden AA, Novak EK, Zhen L, Rusiniak ME, Eicher EM, Robinson MS, Gorin MB, Swank RT (1999) The beta3A subunit gene (Ap3b1) of the AP-3 adaptor complex is altered in the mouse hypopigmentation mutant pearl, a model for Hermansky-Pudlak syndrome and night blindness. Hum Mol Genet 8:323–330

Ferre J, Silva FJ, Real MD, Mensua JL (1986) Pigment patterns in mutants affecting the biosynthesis of pteridines and xanthommatin in *Drosophila melanogaster*. Biochem Genet 24:545–569

Gallusser A, Kirchhausen T (1993) The beta 1 and beta 2 subunits of the AP complexes are the clathrin coat assembly components. EMBO J 12:5237–5244

Gonnet GH, Cohen MA, Benner SA (1992) Exhaustive matching of the entire protein sequence database. Science 256:1443–1445

Gonzalez-Gaitan M, Jäckle H (1997) Role of Drosophila alpha-adaptin in presynaptic vesicle recycling. Cell 88:767–776

Greenberg M, DeTulleo L, Rapoport I, Skowronski J, Kirchhausen T (1998) A dileucine motif in HIV-1 Nef is essential for sorting into clathrin- coated pits and for downregulation of CD4. Curr Biol 8:1239–1242

Hirst J, Robinson MS (1998) Clathrin and adaptors. Biochim Biophys Acta 1404:173–193

Kantheti P, Qiao X, Diaz ME, Peden AA, Meyer GE, Carskadon SL, Kapfhamer D, Sufalko D, Robinson MS, Noebels JL, Burmeister M (1998) Mutation in AP-3 delta in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. Neuron 21:111–122

Kirchhausen T, Bonifacino JS, Riezman H (1997) Linking cargo to vesicle formation: receptor tail interactions with coat proteins. Curr Opin Cell Biol 9:488–495

Kuge O, Hara Kuge S, Orci L, Ravazzola M, Amherdt M, Tanigawa G, Wieland FT, Rothman JE (1993) zeta-COP, a subunit of coatomer, is required for COP-coated vesicle assembly. J Cell Biol 123:1727–1734

Le Borgne R, Alconada A, Bauer U, Hoflack B (1998) The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins. J Biol Chem 273:29451–29461

Lee J, Jongeward GD, Sternberg PW (1994) *unc-101*, a gene required for many aspects of *Caenorhabditis elegans* development and behavior, encodes a clathrin-associated protein. Genes Dev 8:60–73

Lewin DA, Mellman I (1998) Sorting out adaptors. Biochim Biophys Acta 1401:129–145

Lindsley DL, Zimm GG (1992) The genome of *Drosophila melanogaster*. Academic Press, New York

Lloyd V, Ramaswami M, Krämer H (1998) Not just pretty eyes: Drosophila eye-colour mutations and lysosomal delivery. Trends Cell Biol 8:257–259

Mohr OL (1927) Carmine, a new sex-linked eye color in *Drosophila melanogaster*. Z Indukt Abstamm Vererb Lehre 45:403–405

Nakayama Y, Goebl M, O'Brine Greco B, Lemmon S, Pingchang Chow E, Kirchhausen T (1991) The medium chains of the mammalian clathrin-associated proteins have a homolog in yeast. Eur J Biochem 202:569–574

Nolte DJ (1950) The eye pigmentary system of Drosophila: the pigment cells. J Genet 50:79–99

Ohno H, Stewart J, Fournier MC, Bosshart H, Rhee I, Miyatake S, Saito T, Gallusser A, Kirchhausen T, Bonifacino JS (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science 269:1872–1875

Ohno H, Fournier MC, Poy G, Bonifacino JS (1996) Structural determinants of interaction of tyrosine-based sorting signals with the adaptor medium chains. J Biol Chem 271:29009–29015

Ohno H, Aguilar RC, Yeh D, Taura D, Saito T, Bonifacino JS (1998) The medium subunits of adaptor complexes recognize distinct but overlapping sets of tyrosine-based sorting signals. J Biol Chem 273:25915–25921

Ooi CE, Moreira JE, Dell'Angelica EC, Poy G, Wassarman DA, Bonifacino JS (1997) Altered expression of a novel adaptin leads to defective pigment granule biogenesis in the *Drosophila* eye color mutant *garnet*. EMBO J 16:4508–4518

Owen DJ, Evans PR (1998) A structural explanation for the recognition of tyrosine-based endocytotic signals. Science 282:1327–1332

Page LJ, Robinson MS (1995) Targeting signals and subunit interactions in coated vesicle adaptor complexes. J Cell Biol 131:619–630

Panek HR, Stepp JD, Engle HM, Marks KM, Tan PK, Lemmon SK, Robinson LC (1997) Suppressors of YCK-encoded yeast casein kinase 1 deficiency define the four subunits of a novel clathrin AP-like complex. EMBO J 16:4194–4204

Pevsner J, Volknandt W, Wong BR, Scheller RH (1994) Two rat homologs of clathrin-associated adaptor proteins. Gene 146:279–283

Phan HL, Finlay JA, Chu DS, Tan PK, Kirchhausen T, Payne GS (1994) The *Saccharomyces cerevisiae* APS1 gene encodes a homolog of the small subunit of the mammalian clathrin AP-1 complex: evidence for functional interaction with clathrin at the Golgi complex. EMBO J 7:1706–1717

Phillips JP, Forrest HS (1980) Ommochromes and pteridines. In: Ashburner M, Wright TRF (eds) Genetics and biology of *Drosophila*, vol 2d. Academic Press, New York, pp 541–617

Radice P, Pensotti V, Jones C, Perry H, Pierotti MA, Tunnacliffe A (1995) The human archain gene, *ARCN1*, has highly conserved homologs in rice and Drosophila. Genomics 26:101–106

Rapoport I, Miyazaki M, Boll W, Duckworth B, Cantley LC, Shoelson S, Kirchhausen T (1997) Regulatory interactions in the recognition of endocytic sorting signals by AP-2 complexes. EMBO J 16:2240–2250

Rapoport I, Chen YC, Cupers P, Shoelson SE, Kirchhausen T (1998) Dileucine-based sorting signals bind to the beta chain of AP-1 at a site distinct and regulated differently from the tyrosine-based motif-binding site. EMBO J 17:2148–2155

Robinson MS (1993) Assembly and targeting of adaptin chimeras in transfected cells. J Cell Biol 123:67–77

Rothman JE, Wieland FT (1996) Protein sorting by transport vesicles. Science 272:227–234

Schekman R, Orci L (1996) Coat proteins and vesicle budding. Science 271:1526–1533

Schmid SL (1997) Clathrin-coated vesicle formation and protein sorting: an integrated process. Annu rev Biochem 66:511–548

Shestopal SA, Makunin IV, Belyaeva ES, Ashburner M, Zhimulev IF (1997) Molecular characterization of the *deep orange* (*dor*) gene of *Drosophila melanogaster*. Mol Gen Genet 253:642–648

Shih W, Gallusser A, Kirchhausen T (1995) A clathrin-binding site in the hinge of the beta 2 chain of mammalian AP-2 complexes. J Biol Chem 270:31083–31090

Simpson F, Bright NA, West MA, Newman LS, Darnell RB, Robinson MS (1996) A novel adaptor-related protein complex. J Cell Biol 133:749–760

Simpson F, Peden AA, Christopoulou L, Robinson MS (1997) Characterization of the adaptor-related protein complex, AP-3. J Cell Biol 137:835–845

Stephens DJ, Crump CM, Clarke AR, Banting G (1997) Serine 331 and tyrosine 333 are both involved in the interaction between the cytosolic domain of TGN38 and the μ2 subunit of the AP2 clathrin adaptor complex. J Biol Chem 272:14104–14109

Stepp JD, Huang K, Lemmon SK (1997) The yeast AP-3 complex is essential for the efficient delivery of alkaline phosphatase by the alternate pathway to the vacuole. J Cell Biol 139:1761–1774

Tebar F, Sorkina T, Sorkin A, Ericsson M, Kirchhausen (1996) Eps 15 is a component of clathrin-coated pits and vesicles and is located at the rim of coated pits. J Biol Chem 271:28727–28730

Thurieau C, Brosius J, Burne C, Jolles P, Keen JH, Mattaliano RJ, Chow EP, Ramachandran KL, Kirchhausen T (1988) Molecular cloning and complete amino acid sequence of AP50, an assembly protein associated with clathrin-coated vesicles. DNA 7:663–669

Vowels JJ, Payne GS (1998) A dileucine-like sorting signal directs transport into an AP-3-dependent, clathrin-independent pathway to the yeast vacuole. EMBO J 17:2482–2493

Warner TS, Sinclair DA, Fitzpatrick KA, Singh M, Devlin RH, Honda BM (1998) The *light* gene of *Drosophila melanogaster* encodes a homologue of *VPS41*, a yeast gene involved in cellular-protein trafficking. Genome 41:236–243

Wu MN, Bellen HJ (1997) Genetic dissection of synaptic transmission in Drosophila. Curr Opin Neurobiol 5:624–630

Zizioli D, Meyer C, Guhde G, Saftig P, von Figura K, Schu P (1999) Early embryonic death of mice deficient in gamma-adaptin. J Biol Chem 274:5385–5390